Developmental Synergism of Steroidal Estrogens in Sex Determination

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Gonadal sex in the red-eared slider turtle, *Trachemys scripta*, is determined by incubation temperature during embryonic development. Evidence suggests that temperature determines sex by influencing steroid hormone metabolism and/or sensitivity: steroidogenic enzyme inhibitors or exogenous sex steroid hormones and their man-made analogs override (or enhance) temperature effects on sex determination. Specifically, nonaromatizable androgens and aromatase inhibitors induce testis differentiation at female-producing temperatures, whereas aromatizable androgens and estrogens induce ovary differentiation at male-producing temperatures. Moreover, natural estrogens and temperature synergize to produce more females than would be expected if estrogens and temperature had purely additive effects on sex determination. In this study, we use sex reversal of turtle embryos incubated at a male-producing temperature to examine synergism among steroidal estrogens: estrone, 17β-estradiol, and estriol. A low dose of 17β-estradiol (200 ng) showed significant synergism when administered with a single low dose of estriol (10 ng). Likewise, a single low dose of estrone (250 ng) had a synergistic effect when combined with the same low dose of estriol (10 ng). We conclude that the weak natural estrogens estrone and 17β-estradiol synergize with a low dose of the more potent estriol to reverse gonadal sex during the critical period of sexual differentiation. These results suggest that weak environmental estrogens may also synergize with stronger natural estrogens. *Key words*: estradiol, estriol, estrogen, estrone, synergy, temperature-dependent sex determination. *Environ Health Perspect* 107:93–97 (1999). [Online 8 January 1999]
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A number of man-made compounds mimic estrogens, although with a lower potency than natural steroidal estrogens (*1–3*). When considered individually, these chemicals may exist in the environment in concentrations too low to be of concern. In combination, however, low dosages of these same compounds may act synergistically to produce a strong estrogenic response. This low-dose synergy was first shown with polychlorinated biphenyls (PCBs) using an *in vivo* sex-reversal assay in the red-eared slider turtle (*Trachemys scripta*) (*4*). Although the pesticides endosulfan and dieldrin were reported to produce similar synergy using a yeast gene expression system (*5*), subsequent studies failed to replicate this result (*6,7*). Thus, controversy still surrounds environmental estrogens and whether they exhibit synergistic activity. This is an especially important question because low dosages of PCBs and pesticides could pose a health risk through inappropriate activation of estrogen-regulated processes. Indeed, naturally occurring steroidal estrogens play a prominent role in early development and in adult reproductive function through their effects on cell differentiation and cellular organization of the ovary (*8–10*).

The developmental effects of steroidal estrogens are even more pronounced in the red-eared slider turtle because sex steroids have been implicated in the process of temperature-dependent sex determination (*11*). Gonadal sex in this and many other reptiles is determined by embryonic incubation temperature. In this particular species, eggs incubated at constant temperatures below 28.6°C develop as males, eggs incubated at or above 29.6°C develop as females, and increasing temperature within the narrow 28.6–29.6°C range produces increasing proportions of females (*12*). Moreover, temperature has its effect during a critical period of development. If embryos incubating at a male-producing temperature are shifted during the middle third of incubation to a temperature that produces females, the embryos develop as females. This temperature-induced sex reversal, however, is ineffective when the temperature shift occurs after the middle third of incubation. Analagous shift experiments from female- to male-producing temperatures verify that the middle trimester is in fact the temperature-sensitive period (TSP) of development. Incubation temperature also has a quantitative effect on sex determination such that a given period of exposure to a higher temperature produces a stronger feminizing effect than the same period of exposure to a lower temperature (*13,14*).

Importantly, these temperature effects can be overridden (i.e., sex can be reversed) by applying exogenous steroids to the egg during the TSP: estrogens and aromatizable androgens induce female sex determination at male-producing temperatures and nonaromatizable androgens induce male sex determination at female-producing temperatures (*12,15–21*). Although there is some hypertrophy of oviducts following exposure to estriol or to high doses of 17β-estradiol (estradiol), the ovaries of hormone-determined females are indistinguishable from the ovaries of temperature-determined females (*22,23*). Likewise, the testes of hormone-determined males appear normal even though the phallus is overly developed in dihydrotestosterone-treated turtles (*18*). In addition, the steroidal estrogens estrone, estradiol, and estriol have strong dosage effects that synergize with incubation temperature to induce ovarian differentiation. The potency of these estrogens changes with increasing temperature such that estriol is much more potent than estradiol and estrone at a male-producing incubation temperature, and estrone becomes as potent as estriol with increasing temperature (*23*). Synergy also occurs between certain hydroxylated PCBs (*4*). When applied in combination, these compounds have a much stronger estrogenic effect than would be expected based on their individual effects. Overall, these results suggest that temperature influences sex steroid hormone metabolism and/or sex steroid hormone sensitivity and that synergism is an integral part of TSD.

In this study, we used various combinations of three natural estrogens, estrone, estradiol, and estriol, to examine their synergy in sex reversal of turtle embryos incubated at a male-producing temperature. Results of this and previous studies elucidate a possible mechanism contributing to synergy between steroidal estrogens and incubation temperature. The synergistic effects among these natural estrogens not only improve our understanding of TSD

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Table 1. Estrogen treatments and resulting sex ratios of hatchling turtles after incubation at a male-producing temperature

Group	Estrone	Estradiol	Estriol	No.	Male	Female	Percent female
Control	–	–	–	29	29	0	0
1	0.01	–	–	29	27	2	6.90
2	0.10	–	–	27	22	5	18.52
3	0.25	–	–	28	14	14	50.00
4	0.40	–	–	27	15	12	44.44
5	1.0	–	–	29	0	29	100.00
6	–	0.075	–	26	22	4	15.38
7	–	0.200	–	28	18	10	35.71
8	–	0.400	–	29	10	19	65.52
9	–	1.000	–	28	0	28	100.00
10	–	5.000	–	30	0	30	100.00
11	–	–	0.01	30	26	4	13.33
12	–	–	0.02	29	23	6	20.69
13	–	–	0.03	28	13	15	53.57
14	–	–	0.05	30	7	23	76.67
15	–	–	1.00	27	1	26	96.30
16	0.01	0.075	–	29	26	3	10.34
17	0.10	0.075	–	30	22	8	26.67
18	0.25	0.075	–	27	11	16	59.26
19	–	0.075	0.01	29	18	11	37.93
20	–	0.075	0.02	28	16	12	42.86
21	–	0.075	0.03	25	10	15	60.00
22	–	0.200	0.01	26	8	18	69.23
23	–	0.400	0.01	29	8	21	72.41
24	0.01	–	0.01	30	27	3	10.00
25	0.10	–	0.01	30	21	9	30.00
26	0.25	–	0.01	30	6	24	80.00
27	0.01	0.075	0.01	29	19	10	34.48
28	0.10	0.075	0.02	28	12	16	57.14
29	0.25	0.075	0.03	30	8	22	73.33
30	0.01	0.200	–	30	14	16	53.33

All estrogen treatments (nanograms) were delivered in a total of 5 µl ethanol. Controls were treated with ethanol only.

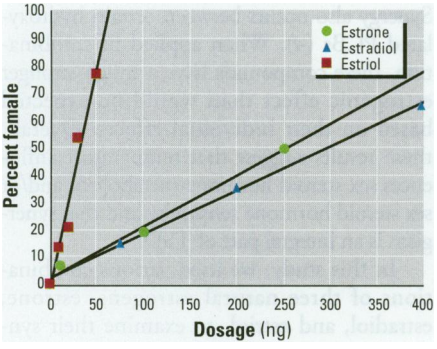

Figure 1. Sex ratios of hatchling turtles incubated at a male-producing temperature after treatment with increasing dosages of three steroidal estrogens, estrone, estradiol, and estriol, each applied individually. Separate linear regressions are shown for each steroid using the sex ratios presented (i.e., not over the entire range of doses tested).

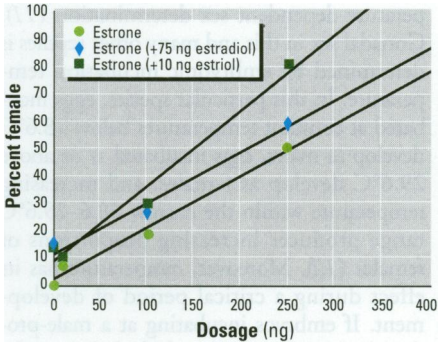

Figure 2. Sex ratios of hatchling turtles incubated at a male-producing temperature after treatment with increasing dosages of estrone alone and with the same estrone dosages combined with 75 ng estradiol or 10 ng estriol. Separate linear regressions are shown for the single and dual hormone treatments using the sex ratios presented (i.e., not over the entire range of doses tested).

but also suggests that estrogens (or temperature) may produce synergistic responses when combined with low doses of synthetic compounds that are weakly estrogenic.

Materials and Methods

Freshly laid eggs from the red-eared slider turtle, *Trachemys scripta*, were obtained commercially (Robert Kliebert, Hammond, LA). After transport to the laboratory, the eggs were held at room temperature until we established embryo viability by candling them. The eggs were then placed in groups of 30 in covered trays containing moistened vermiculite (vermiculite:water, 1:1) and placed in one incubator (Precision, Chicago, IL) programmed to maintain a constant temperature of 26.0°C. Temperature fluctuations were monitored daily by checking the incubator program, two calibrated thermometers, and a computerized data logger (HOBO temperature mini-logger, Onset Computer Corporation, Pocasset, MA). Embryo development was monitored by candling and dissecting representative eggs to verify characteristics specific to particular developmental stages (*24*). All animal handling and treatments are performed in accordance with the Institutional Animal Care and Use Committee guidelines for the University of Texas at Austin.

Estrogens were dissolved in 5 µl of 95% ethanol and applied to the vascularized portion of the egg shell at embryonic Stage 17, which corresponds to the beginning of the TSP (*13*). Each treatment group initially contained 30 eggs. Single treatments with estrone (10, 100, 250, 400, or 1,000 ng), estradiol (75, 200, 400, 1,000, or 5,000 ng), and estriol (10, 20, 30, 50, or 1,000 ng), and a series of combinations of these hormones were used to test for synergistic interactions among natural estrogens (see Table 1). Hormone combinations were premixed and delivered in a single 5-µl application, as were individual hormones. The control group (n = 30) was treated with 5 µl of ethanol. After treatment, all eggs were returned to the incubator until they hatched. During the entire period of incubation, egg trays were rotated daily from shelf to shelf to avoid any possible effects of a temperature gradient within the incubator.

Within 2 weeks of hatching, turtles were anesthetized, decapitated, and examined in a blind study, so that treatments were unknown until all data had been collected. Gonadal sex and oviducts were examined under a dissection microscope by two different researchers and recorded. At hatching, red-eared slider turtles have well-differentiated gonads that can be reliably identified as ovaries or testes using this method (*13*). Briefly, ovaries are long and thin, lack vascularization, and are colorless, whereas testes are shorter, rounder, and vascularized, and have visible sex cords and a yellowish color. The presence and development (i.e., whether normal, hypertrophied, or regressed) of oviducts was also noted. Several gonads from each of the groups of 30 were removed and assessed histologically. In all cases, macroscopic and histological diagnosis of sex were consistent.

Two sex-reversal experiments were designed to test for synergy among natural estrogens. We first tested for synergism between pairs of estrogens. In the second study, we tested for synergism among all three estrogens when applied simultaneously. Specifically, we compared the observed sex ratio with combined treatments to the expected sex ratio if the hormones act in an

additive manner: synergy occurs if the observed sex ratio is significantly greater than the expected sex ratio. The expected proportion (P) of females for combined hormone treatments under the additive model was derived as follows, with $P_{estrone}$ as the proportion of females treated with a given dosage of estrone; $P_{estradiol}$ as the proportion of females treated with a given dosage of estradiol; and $P_{estriol}$ as the proportion of females treated with a given dosage of estriol.

Thus, the null hypothesis for the additive effect of the hormones in combination is

$$P_{estrone + estradiol + estriol} = P_{estrone} + P_{estradiol}(1 - P_{estrone}) + P_{estriol}\{1 - [P_{estrone} + P_{estradiol}(1 - P_{estrone})]\}. \quad (1)$$

The expected proportion of females for each of the combinations of estrogens was derived from Equation 1 and sample Ps from the appropriate single hormone-dosage groups. Chi-square (χ^2) tests were then used to compare observed to expected sex ratios.

Results

Overall, the results for single hormone treatments were similar to those determined for the same estrogens at the same temperature in a previous study (*23*). Sex reversal occurred in a dose-dependent manner with each estrogen alone (Fig. 1, also see Table 1). Specifically, we found that estrone and estradiol display one-tenth the potency of estriol in sex reversal. This difference was clearly evident when comparing the dosage at which 50% of the embryos were sex reversed. Sex ratio data for the estrone dosage of 0.4 µg was discarded, as the value was an outlier [the observed sex ratio was too low in comparison with the pattern displayed in the current data set and the expected sex ratio based on Crews et al. (*23*)].

Sex reversal also occurred in a dose-dependent manner with estrogens in various paired combinations (Figs. 2–4; also see Table 1). Two of the dual estrogen treatments produced significant synergy (Table 2). Combined dosages of 10 ng estriol + 200 ng estradiol had a greater observed than expected sex ratio ($\chi^2 = 6.58$; $p<0.05$), as did 10 ng estriol + 250 ng estrone ($\chi^2 = 6.65$; $p<0.05$). In contrast, sex ratios for the other paired estrogen treatments were consistent with the additive model of hormone effects.

Although increasing dosages in the triple estrogen treatment also produced increasing proportions of females (see Table 1), there was no evidence of synergism in any of the combined treatments using all three hormones.

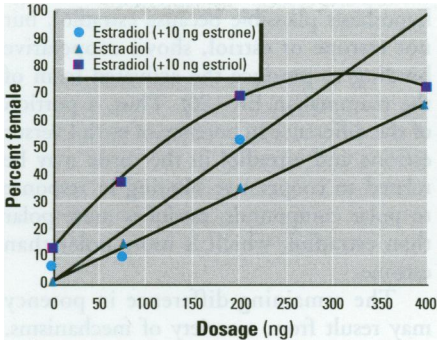

Figure 3. Sex ratios of hatchling turtles incubated at a male-producing temperature after treatment with increasing dosages of estradiol alone and with the same estradiol dosages combined with 10 ng estrone or 10 ng estriol. Separate regressions are shown for the single and dual hormone treatments using the sex ratios presented (i.e., not over the entire range of doses tested).

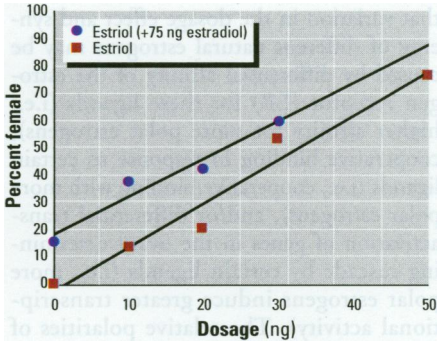

Figure 4. Sex ratios of hatchling turtles incubated at a male-producing temperature after treatment with increasing dosages of estriol alone and with the same estriol dosages combined with 75 ng estradiol. Separate regressions are shown for the single and dual hormone treatments using the sex ratios presented (i.e., not over the entire range of doses tested).

Table 2. Pairwise comparisons of dual estrogen treatments of hatchling turtles after incubation at a male-producing temperature

	Observed		Expected		
Treatment (ng)	F	M	F	M	χ^2
Estradiol (0.075) + estrone (0.01)	3	26	6.2	22.8	2.09
Estradiol (0.075) + estrone (0.10)	8	22	9.3	20.7	0.26
Estradiol (0.075) + estrone (0.25)	16	11	15.6	11.4	0.02
Estradiol (0.075) + estriol (0.01)	11	18	7.7	21.3	1.92
Estradiol (0.075) + estriol (0.02)	12	16	9.2	18.8	1.27
Estradiol (0.075) + estriol (0.03)	15	10	15.2	9.8	0.01
Estriol (0.01) + estradiol (0.075)	11	18	7.7	21.3	1.92
Estriol (0.01) + estradiol (0.200)	18	8	11.5	14.5	6.58*
Estriol (0.01) + estradiol (0.400)	21	8	20.3	8.7	0.08
Estriol (0.01) + estrone (0.01)	3	27	5.8	24.2	1.67
Estriol (0.01) + estrone (0.10)	9	21	8.8	21.2	0.01
Estriol (0.01) + estrone (0.25)	24	6	17.0	13.0	6.65*
Estrone (0.01) + estradiol (0.75)	3	26	6.2	22.8	2.09
Estrone (0.01) + estradiol (0.20)	16	14	12.0	18.0	2.22

Abbreviations: F, female; M, male.
Comparison of observed to expected sex ratio used to examine synergistic or antagonistic effects of dual estrogen treatments.
*Significant difference in the χ^2 values at a level of $p<0.05$; both significant treatments show a higher observed ratio compared to expected ratio, therefore demonstrating synergy.

The observed sex ratios were consistent with the expected sex ratios, based on the null hypothesis of additive effects of these hormones. There was no difference between the observed (35% female) and expected (32% female) sex ratios when estrone (10 ng), estradiol (75 ng), and estriol (10 ng) were combined in a single treatment ($\chi^2 = 0.16$; $p>0.5$); there was no difference between the observed (57% female) and expected (45% female) sex ratios for the combined estrone (100 ng), estradiol (75 ng), and estriol (20 ng) treatment ($\chi^2 = 1.29$, $p>0.1$); and there was no difference between the observed (73% female) and expected (80% female) sex ratios for the combined estrone (250 ng), estradiol (75 ng), and estriol (30 ng) treatment ($\chi^2 = 0.83$; $p > 0.1$).

Discussion

In this study we demonstrate that estrone, estradiol, and estriol have different dosage effects when administered individually and that certain pairs of these estrogens produce a synergistic effect on sex reversal of turtle embryos incubated at a male-producing temperature. Specifically, we found that estrone and estradiol display one-tenth the potency of estriol. This result is robust because the dosage effects in this study are very similar to the dosage effects produced in an earlier study using the same hormones at the same incubation temperature (*23*). In addition, estriol at a low dose synergizes with the weaker estrogens estrone and estradiol, also at low doses. These results suggest that potent estrogens (i.e., estriol) or temperature may also synergize with low doses of weakly estrogenic man-made compounds. The latter proposition is likely, considering that an admixture of hydroxylated PCBs is much more potent in sex reversal than the same compounds given individually at 10-fold higher doses (*4*).

Although the mechanisms underlying these results are still unknown, we propose

that variation in the dosage effect and synergy of different natural estrogens may be caused by differential affinity of the estrogen receptor (ER) for these ligands (i.e., higher affinity for more polar estrogens), cooperative binding in response to certain ligands (i.e., cooperative binding with more polar estrogens), and/or differential transactivation of genes in the ovary determining cascade by certain ligands (i.e., more polar estrogens induce greater transcriptional activity). The relative polarities of these estrogens are estriol > estradiol > estrone. If any of these mechanisms occur, they could result in synergistic activity at low doses (*25*).

The first hypothesis, namely, that the turtle ER has a higher affinity for estriol than estrone or estradiol, has a precedent. The mammalian ER, for example, has a much higher *in vitro* affinity for estradiol than for either estrone or estriol, which is reflected in different pharmacokinetic effects *in vivo* (*26*). Another possibility centers around ER expression, where binding characteristics may differ in response to the concentration of estrogens and their receptors. Proposed future biochemical assays of the purified turtle receptor will directly address these hypotheses in the red-eared slider.

Another possible mechanism for the high individual potency and apparent synergy of estriol in sex reversal may be found in the effective concentrations of the three estrogens. A sex-steroid binding protein (SSBP) has been identified in the painted turtle (*Chrysemys picta*) that binds to estradiol but does not bind to estrone or estriol (*27*). If this SSBP binds estradiol away from the cell, but estriol is still unbound, the estriol may have greater effect due to ready access to the cellular receptors. This does not, however, explain the greater potency of estriol relative to estrone, unless there are species differences in the SSBP.

The sex-reversing and synergistic effects of estriol could also be explained by cooperative ligand binding of estriol, but not estrone or estradiol, to the ER. Indirect evidence suggests that cooperative binding of estriol, but not estrone or estradiol, contributes to the larger developmental effect of estriol in the turtle. Indeed, a low dose of estradiol synergizes with a single low dose of estriol. This increase may reflect an estriol-facilitated binding of estradiol (and of more estriol when considering a single hormone treatment) to form the activated receptor dimer. Estriol-facilitated binding may also be responsible for the increased sex reversal (greater than additive) when combined with estrone. Again, binding kinetics of the mammalian ER make this hypothesis plausible because estradiol, but not estrone or estriol, shows cooperative binding to produce the activated form of the mammalian ER (*26*). Thus, a portion of the difference in potency of estriol versus estrone and estradiol in the turtle may be related to cooperative binding in response to polar compounds: estriol is more polar than estradiol, which is more polar than estrone.

The remaining difference in potency may result from a variety of mechanisms. For example, receptors activated by different estrogens may have different binding affinities for the same estrogen responsive element (ERE) and/or different transactivational effects on transcription once bound to the same ERE (*28*). In fact, two separate activational domains, AF-1 and AF-2, on the ER can respond in different ways to ligand binding (*29*). In general, AF-1 transactivation is not dependent upon ligand binding, whereas AF-2 transactivation is induced by ligand binding (*30,31*). In a yeast expression assay, Tran et al. (*32*) demonstrated that hydroxylated PCBs were less dependent on the AF-1 transactivation site for estrogenic activity than estradiol was. The sex reversal effects of estriol may also be due to its ability to induce estrogen-mediated processes through synergistic interactions between AF-1 and AF-2 during transactivation.

Although clearly important in temperature-dependent sex determination, ER expression is not induced by incubation temperature because complete estrogen-induced sex reversal can occur even at temperatures that normally produce all males. If receptors were limiting, even high dosages of estrogen would not induce sex reversal of all embryos. Though there is no detectable difference in uptake or binding of radiolabeled estrogens to the adrenal–kidney–gonad complex of embryos incubated at a male- versus a female-producing incubation temperature (*33*), there is a difference in ER mRNA expression. When compared to embryos from a male-producing incubation temperature, embryos from a female-producing incubation temperature show higher ER mRNA levels in the urogenital ridge prior to gonadal differentiation (*34*).

Another hypothesis is that temperature directly (or indirectly through an upstream regulatory protein) up regulates expression of steroidogenic enzymes that in turn produce a dichotomous milieu of sex steroids, which then induce gonadal differentiation. The prime candidate for this key regulatory enzyme has been aromatase, the enzyme that catalyzes the production of estrogens from androgens (*35*). There is some support for this hypothesis in that aromatase inhibitors block the production of females at female- or mixed sex ratio-producing temperatures and also block the feminizing effect of aromatizable androgens at male-producing temperatures (*20,36–38*). There are no detectable differences, however, in gonadal aromatase activity or gonadal aromatase mRNA levels either before or during the TSP in turtles incubated at male- versus female-producing temperatures (*39–43*). Furthermore, differences in aromatase expression toward the end of the TSP may be the result, rather than the cause, of gonadal differentiation (*39–41*). While aromatase may be an important component in the process of TSD, there is no clear evidence that expression of this enzyme is induced by temperature.

Considering the results from our current and previous studies of the red-eared slider, expression of 16α-hydroxylase may be developmentally regulated by temperature. This enzyme regulates the conversion of estrone, and by default estradiol, into the more potent estriol. Interestingly, all three estrogens used in the current study synergize with incubation temperature to produce more females than expected if temperature and estrogens had purely additive effects on ovarian differentiation (*23*). Up regulation of 16α-hydroxylase by increasing temperatures would explain the increase in potency of estrone and estradiol at higher temperatures through their greater conversion to estriol. Specifically, the dosage effect of estrone becomes equivalent to that of estriol at a temperature that normally produces approximately 20% females, while estradiol becomes as potent as estriol at yet higher temperatures. Specific inhibitors for 16α-hydroxylase could be used to examine their effect on sex reversal. The effect of temperature on expression of this enzyme must also be determined to test this hypothesis.

Besides the important clues it may give to mechanisms of temperature and estrogens in TSD, this study also gives credence to the proposition that strong natural estrogens at low doses may synergize with low doses of weak natural and man-made estrogens. Because environmental compounds that exhibit estrogenic effects have potencies far less than that of estradiol (*2,3*), there are questions as to whether these chemicals have any appreciable effect in nature (*44*). Two recent studies (*6,45*) have questioned the reported synergy found with combined synthetic compounds (*5*). Dosage is critical to observing the combined effect, as synergy was only evident at the low end of the test range (*46*). The lowest combined doses with dieldrin used by Ashby et al. (*45*) are in a range that

Arnold et al. (*5*) showed to have an already strong single-compound effect. If the dose of one of the compounds tested produces maximal or near-maximal activity, synergy will not be detected. Although a low dose of estriol combined with low doses of estrone and estradiol showed synergistic sex reversal of turtle embryos in our study, the magnitude of the effect was relatively small compared to the synergy observed between estrogens and temperature or between hydroxylated PCBs.

If synergistic responses to natural or synthetic estrogens are attributable to specific properties of TSD, understanding such a mechanism will be important in explaining developmental effects of estrogens. As demonstrated here and in previous work (*23*), certain aspects of TSD (e.g., temperature, ER expression, steroidogenic enzyme expression) will determine the potency of estrogenic compounds. These characteristics are critical to examining whether low doses of man-made chemicals have synergistic effects (*47,48*). In any case, exposure to estrogenic compounds, whether man-made or natural, at pivotal stages of development may result in irreversible changes such as the feminization of male carp during gonadal differentiation (*49,50*) or the complete reversal of gonadal sex in reptiles with TSD.

References and Notes

1. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380–383 (1994).
2. McLachlan JA. Functional toxicology: a new approach to detect biologically active xenobiotics. Environ Health Perspect 101(5):386–387 (1993).
3. Korach KS, Sarver P, Chae K, McLachlan JA, McKinney JD. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. Mol Pharmacol 33:120–126 (1988).
4. Bergeron JM, Crews D, McLachlan JA. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 102:780–781 (1994).
5. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette LJ Jr, McLachlan JA. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 272:1489–1492 (1996).
6. Ramamoorthy K, Wang F, Chen I-C, Safe S, Norris JD, McDonnell DP, Gaido KW, Bocchinfuso WP, Korach KS. Potency of combined estrogens. Science 275:405 (1997).
7. McLachlan JA. Synergistic effect of environmental estrogens: report withdrawn. Science 277:462 (1997).
8. George FW, Wilson JD. Sex determination and differentiation. In: The Physiology of Reproduction (Knobil E, Neill J, eds). 2nd ed. New York:Raven Press, 1994;3–28.
9. Scheib D. Effects and role of estrogens in avian gonadal differentiation. Differentiation 23 (suppl):S87–S92 (1983).
10. Gasc J-M. Estrogen target cells in gonads of the chicken embryo during sexual differentiation. J Embryol Exp Morphol 55:331–342 (1980).
11. Crews D. Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zool Sci 13:1–13 (1996).
12. Crews D, Bergeron JM, Flores D, Bull JJ, Skipper JK, Tousignant A, Wibbels T. Temperature-dependent sex determination: proximate mechanisms, functional outcomes, and practical applications. Dev Genetics 15:297–312 (1994).
13. Wibbels T, Bull JJ, Crews D. Chronology and morphology of temperature-dependent sex determination. J Exp Zool 260:371–381 (1991).
14. Georges A, Limpus C, Stoutjesdijk R. Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. J Exp Zool 270:432–444 (1994).
15. Bull JJ, Gutzke WHN, Crews D. Sex reversal by estradiol in three reptilian orders. Gen Comp Endocrinol 70:425–428 (1988).
16. Crews D, Bull JJ, Wibbels T. Estrogen and sex reversal in turtles: a dose-dependent phenomenon. Gen Comp Endocrinol 81:357–364 (1991).
17. Crews D, Cantú AR, Bergeron JM, Rhen T. The relative effectiveness of androstenedione, testosterone, and estrone, precursors to estradiol, in sex reversal in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 100:119–127 (1995).
18. Crews D, Cantú AR, Bergeron JM. Temperature and non-aromatizable androgens: a common pathway in male sex determination in a turtle with temperature-dependent sex determination? J Endocrinol 149:457–463 (1996).
19. Wibbels T, Crews D. Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD. Gen Comp Endocrinol 100:93–100 (1995).
20. Wibbels T, Crews D. Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with TSD. J Endocrinol 141:295–299 (1994).
21. Wibbels T, Bull JJ, Crews D. Synergism between temperature and estradiol: a common pathway in turtle sex determination? J Exp Zool 260:130–134 (1991).
22. Wibbels T, Gideon P, Bull JJ, Crews D. Estrogen- and temperature-induced medullary regression during gonadal differentiation in a turtle. Differentiation 53:149–154 (1993).
23. Crews D, Cantú AR, Rhen T, Vohra R. The relative effectiveness of estrone, estradiol-17β, and estriol in sex reversal in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 102:317–326 (1996).
24. Yntema CL. A series of stages in the embryonic development of *Chelydra serpentina*. J Morphol 125:219–252 (1968).
25. Simons SS Jr. Environmental estrogens: can two "alrights" make a wrong? Science 272:1451 (1996).
26. Sasson S, Notides AC. Estriol and estrone interaction with the estrogen receptor. J Biol Chem 258:8113–8117 (1983).
27. Salhanick ACR, Callard IP. A sex-steroid binding protein in the plasma of the freshwater turtle, *Chrysemys picta*. Gen Comp Endocrinol 42:163–166 (1980).
28. Johnston SD, Liu X, Zuo F, Eisenbraun TL, Wiley SR, Kraus RJ, Mertz JE. Estrogen-related receptor α1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. Mol Endocrinol 11:342–352 (1997).
29. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365 (1997).
30. Webster NJG, Green S, Jin JR, Chambon P. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. Cell 54:199–207 (1997).
31. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 51:941–951 (1987).
32. Tran DQ, Yin L, Guillette LJ Jr, McLachlan JA, Arnold SF. Unpublished data.
33. Gahr M, Wibbels T, Crews D. Site of estrogen uptake in embryonic *Trachemys scripta*, a turtle with temperature-dependent sex determination. Biol Reprod 46:458–463 (1992).
34. Bergeron JM, Gahr M, Horan K, Wibbels T, Crews D. Cloning and *in situ* hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. Dev Growth Differ 40:243–254 (1997).
35. Pieau C, Girondot M, Desvages G, Dorizzi M, Richard-Mercier N, Zaborski P. Environmental control of gonadal differentiation. In: The Differences Between the Sexes (Short RV, Balaban E, eds). Cambridge:Cambridge University Press, 1994;433–448.
36. Crews, D, Bergeron JM. Role of reductase and aromatase in sex determination in the red-eared slider turtle (*Trachemys scripta*), a turtle with temperature-dependent sex determination. J Endocrinol 143:279–289 (1994).
37. Dorizzi M, Richard-Mercier N, Desvages G, Girondot M, Pieau C. Masculinization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination. Differentiation 47:9–17 (1994).
38. Rhen T, Lang JW. Temperature-dependent sex determination in the snapping turtle: manipulation of the embryonic sex steroid environment. Gen Comp Endocrinol 96:243–254 (1994).
39. Desvages G, Pieau C. Steroid metabolism in gonads of turtle embryos as a function of the incubation temperature of eggs. J Steroid Biochem Mol Biol 39:203–213 (1991).
40. Desvages G, Pieau C. Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. J Steroid Biochem Mol Biol 41:851–853 (1992).
41. Desvages G, Pieau C. Time required for temperature-induced changes in gonadal aromatase activity and related gonadal structures in turtle embryos. Differentiation 52:13–18 (1992).
42. Smith CA, Elf PK, Lang JW, Joss JMP. Aromatase enzyme activity during gonadal sex differentiation in alligator embryos. Differentiation 58:281–290 (1995).
43. Willingham EJ, Crews D. Sex-reversal effects of environmentally relevant xenobiotic concentrations on the red-eared slider turtle, a species with temperature-dependent sex determination. Gen Comp Endocrinol (in press).
44. Safe SH. Environmental and dietary estrogens and human health: is there a problem? Environ Health Perspect 103:346–351 (1995).
45. Ashby J, Lefevre PA, Odum J, Harris CA, Routledge EJ, Sumpter JP. Synergy between synthetic oestrogens? Nature 385:494 (1997).
46. McLachlan JA, Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette LJ Jr. Potency of combined estrogenic pesticides: response. Science 275:405–406 (1997).
47. Sheehan DM, Willingham E, Gaylor D, Bergeron JM, Crews D. No threshold dose for estradiol-induced sex reversal of turtle embryos: how little is too much? Environ Health Perspect (in press).
48. vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Nat Acad Sci USA 94:2056–2061 (1997).
49. Gimeno S, Gerritsen A, Bowmer T, Komen H. Feminization of male carp. Nature 384:221–222 (1996).
50. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).